



POTENTIATING THE ITALIAN CAPACITY FOR STRUCTURAL BIOLOGY SERVICES IN INSTRUCT-ERIC

PROTOCOL FOR CA2 PURIFICATION FROM MAMMALIAN CELLS

IO2.2.1: Empowering mammalian expression at Instruct-IT

Activity 2.2: Expanding the mammalian expression platform for in-cell NMR

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Period: B9 (March - April 2024)

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Call for proposals MUR n. 3264 in date 28/12/2021

Mission 4 – Education and Research Component 2: from research to business

Investment 3.1 Fund for the realisation of an integrated system of research and innovation infrastructures

Action 3.1.1 Creation of new research infrastructures, strengthening of existing ones and their networking for Scientific Excellence under Horizon Europe

RI : INSTRUCT-ERIC – Integrated Structural Biology Infrastructure

ESFRI domain: Health and Food

RI at high priority

Funding Admission DD MUR n. 115 in date 21/06/2022

Starting Date: 1 November 2022

Duration of the project: 30 month

ID Proposal: IR0000009

CUP: B53C22001790006

Protocol for CA2 purification from mammalian cells

- CA2 was transiently expressed in HEK293T cells (see mammalian expression protocol) and purified by affinity chromatography.
- Transfected cells from three identical T75 flasks were lysed in 150 μ L PBS each by the freeze–thaw method and centrifuged at 14000 rpm for 1 h at 4°C to spin down cell debris.
- The resulting supernatants were pooled together and diluted with binding buffer (20 mM Tris, pH 8) to a final volume of 3 mL.
- The lysate was loaded on a 1 mL Ni-NTA column equilibrated with binding buffer, and washed with 3 mL of binding buffer.
- Elution was carried out with 3 mL steps of increasing concentrations of imidazole in binding buffer (Table 1).

Fraction	Imidazole (mM)
Wash 1	0
Elution 1	10
Elution 2	20
Elution 3	30
Elution 4	40
Elution 5	50
Elution 6	70
Elution 7	100
Elution 8	200
Elution 9	500

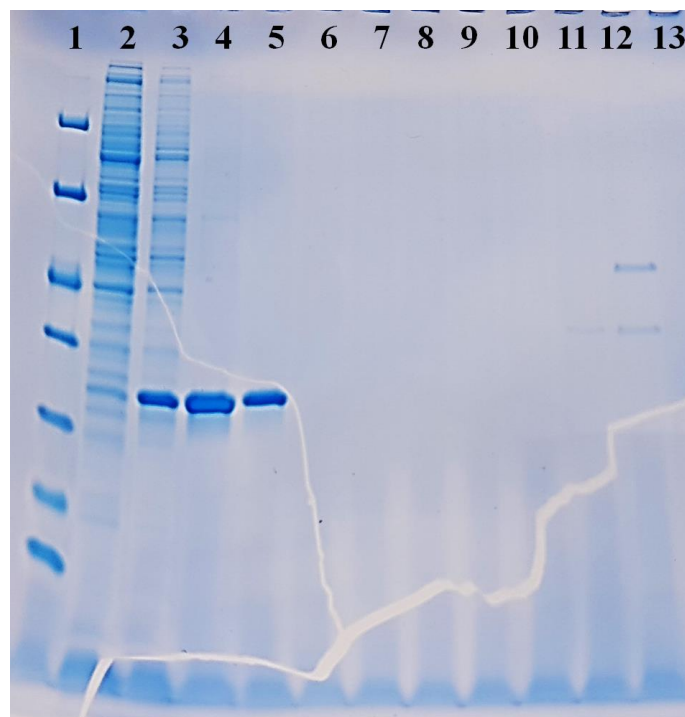


Figure 1. SDS-PAGE of the elution fractions from the Ni-NTA column. Native CA2 (29 kDa) runs between the 4th and the 5th bands of the MW marker. Lanes from left to rights: 1. MW marker; 2. Flow-through; 3. Wash 1; 4. Elution 1; 5. Elution 2; 6. Elution 3; 7. Elution 4; 8. Elution 5; 9. Elution 6; 10. Elution 7; 11 Elution 8; 12. Elution 9

- CA2 was eluted at high purity in the 10 mM and 20 mM imidazole fractions (Figure 1). Henceforth, a simplified elution step can be implemented where the protein is directly eluted with 20 mM imidazole following the wash.
- Following elution, the protein buffer was exchanged 10 mM HEPES, pH 6.8, using a centrifugal concentrator, and subsequently concentrated to 500 μ L. The protein purity was >95% from the SDS-PAGE.
- The CA2 concentration in the 500 μ L sample was 150 μ M, measured by UV-VIS absorbance at 280 nm.

- The 1D ^1H NMR spectrum revealed that the protein is correctly folded (Figure 2).

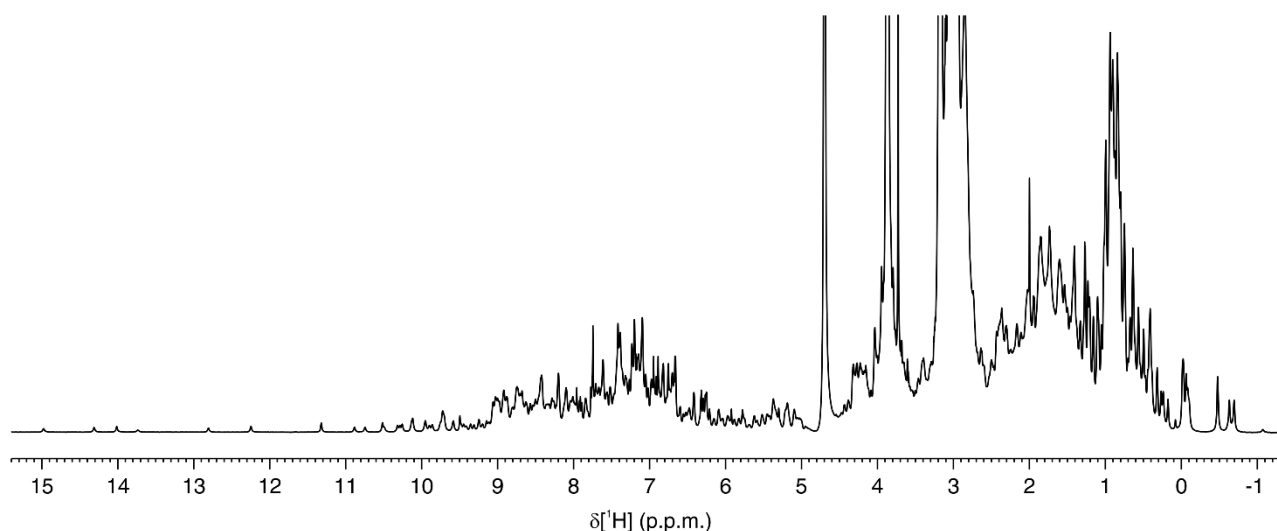


Figure 1. 1D ^1H NMR spectrum of purified CA2 in 10 mM HEPES buffer, pH 6.8. Signal dispersion in the methyl region (between 0 and -1 ppm) and in the amide region (between 6 and 11 ppm) reveals that the protein is well folded. The presence of signals in the downfield region between 11 and 15 ppm, which arise from histidine NH protons in the active site, reveal that the protein is correctly metallated with Zn^{2+} .

- The calculated protein yield from three T75 flasks was ~2.1 mg. As each flask contains 20 mL of medium, this corresponds to a yield of ~35 mg per liter of culture.

